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Original Article

The effects of vitamin D_3 supplementation on some metabolic and inflammatory markers in diabetic nephropathy patients with marginal status of vitamin D: A randomized double blind placebo controlled clinical trial

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ABSTRACT

Aims: Diabetic nephropathy is known to be an independent risk factor in the progression of renal and cardiovascular disorders. Due to the association between vitamin D deficiency and diabetic nephropathy, vitamin D deficiency in the diabetic nephropathy population, this study conducted to examine the effects of Vitamin D_3 on metabolic and inflammatory parameters in patients with diabetic nephropathy.
Methods: This eight-week, randomized, double-blind, placebo-controlled trial was carried out on 50 diabetic nephropathy patients with marginal status of vitamin D. Participants were randomly assigned to two groups: control and intervention. Participants received a vitamin D3 (50000 IU) supplement weekly on a specific day. Fasting blood samples were collected from all patients at their entry to the study, and eight weeks after intervention.
Results: Analyses showed significance differences in physical activity between the intervention and placebo groups ($P = 0.018$). There were no significant differences between the percentage changes of HbA1c, insulin and, inflammatory parameters such as TNF-α and IL-6 ($P > 0.05$), while the percentage change of FBS was significantly higher in the placebo group compared to the treatment one ($P < 0.0001$). Lower levels of FBS ($P < 0.0001$), insulin ($P < 0.069$), HOMA-IR ($P < 0.001$), TNF-α ($P < 0.002$) and IL-6 ($P < 0.037$) were found after supplementation in treatment group. However, the phosphorous and protein percentage change in urine were lower ($P = 0.07$) and higher ($P = 0.003$) between groups.
Conclusions: It was found that vitamin D supplementation can be regarded as an effective way to prevent the progression of diabetic nephropathy by reducing levels of proteinuria, and inflammatory markers such as TNF-α and IL-6.

1. Introduction

Type 2 diabetes, which is related to increased blood sugar, is a group of metabolic disorders related to changes in lifestyle, diet, and obesity [1]. Diabetes development leads to both macro and microvascular disorders. Diabetic nephropathy is one of the most important microvascular complications of diabetes progression, affecting almost one third of patients [2]. It is defined by elevated

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urine albumin excretion and increased blood pressure, which leads to decreasing glomerular filtration and, finally, kidney failure [3,4]. Diabetic nephropathy is known as an independent risk factor for cardiovascular disorders [3] and is also associated with renal manifestations [5].

Numerous studies suggest that inflammation appears to be one of the most important factors in diabetic nephropathy progression [6]. The number of inflammatory cells in the kidney is closely associated with diabetic nephropathy [7–9]. Elevated concentrations of inflammatory molecules, including inflammatory cytokines, has been documented previously in diabetic patients [10]. Recent studies have shown increased levels of these substances is associated with diabetic nephropathy. Cytokines are a group of polypeptides with low molecular weight [11]. These substances have paracrine, autocrine and juxtacrine effects which together regulate immune and inflammatory responses [11]. Since fibrosis is regarded as one of the most relevant complications of diabetic nephropathy [12], and since inflammation is strongly associated with fibrosis [13], it seems that reduced levels of inflammatory factors have beneficial effects in nephropathy patients. The levels of cytokines, such as TNF-α and interleukins, is increased in diabetic patients [14]. However, recent studies have shown that TNF-α and IL-6 levels are higher in patients with nephropathy compared to diabetic patients without nephropathy [15].

Vitamin D is a fat-soluble molecule which is well-known due to its role in bone mineralization [16]. The association of vitamin D with a wide range of disorders, such as cardiovascular disease, hypertension and diabetes, has been documented previously [17]. Moreover, the association between vitamin D deficiency and diabetic nephropathy has been demonstrated in previous studies. The risk of diabetic nephropathy in type 2 diabetic patients with lower vitamin D is higher, compared to those who have higher vitamin D levels [18]. Besides these, the evidence suggests that vitamin D can promote pancreatic β-cell survival by affecting the influences of cytokines and nuclear transcription factors such as NF-κB which regarded as an important factor in proinflammatory signaling pathway [19].

Therefore, with attention to the inadequate status of vitamin D in the population, particularly in patients with diabetes and kidney disease, the aim of this study was to examine the effects of vitamin D_3 supplementation on metabolic and inflammatory parameters in patients with diabetic nephropathy.

2. Material and methods

Subjects and Study Design: An eight-week, randomized, double-blind, placebo-controlled trial was carried out on 50 diabetic nephropathy patients (TDM2) to study the effects of vitamin D_3 supplementation on metabolic (including blood glucose, insulin, HOMA-IR and HbA1c) and inflammatory markers (including TNF-α and IL-6) in patients with diabetic nephropathy. Convenience sampling was used, and a total of 50 diabetic nephropathy patients were recruited (8 week sampling) in the current study from patients referred to the endocrinology clinic of Imam Reza Hospital in Tabriz. The sample size was calculated based on the formula suggested in previous studies [20]:

$$n = \frac{\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2 \left(s_1^2 + s_2^2\right)^2}{\left(m_1 - m_2\right)^2}$$

Where taking into account the effect of vitamin D on the level of TNF-α as one of the important goals of the study, with a type 1 error of 0.05 and a power of 90%. All participants provided written informed consent before taking part in the study. Inclusion criteria included: age 20–50 years, TDM2 was one of inclusion criteria and none of the patients used insulin, controlled fasting blood sugar glucose (sugar in a blood sample after an overnight fast: FBS) less than 140 mg/dl, BMI range 20–35, diabetic nephropathy stage 3 and 4, albuminuria more than 30 mg/day, with marginal status of vitamin D (more than 15 ng/l and less than 30 ng/l), not taking calcium supplements and vitamin D in the last 3 months, and not taking drugs that affect vitamin D metabolism (such as parathormone, estrogens, and calcitonin) and proteinuria. Exclusion criteria included consumption of calcium and vitamin D supplements during the study, glomerulonephritis (determined by nephrologist), phosphorus more than 4.5 mg/dl (normal range 2.5–4.5 mg/dl) and calcium more than 10 mg/dl (normal range 8.5–10 mg/dl). Participants were randomly assigned to two groups, control and intervention, using random numbers generated by software.

Fasting blood samples were collected for all patients before intervention. During the 8-week intervention, the intervention group received a vitamin D_3 (50000 IU) supplement weekly on a specific day all based on standard guidelines and previous studies [21,22]. At the same time, the control group also received a placebo containing Miglyol oil, of the same color and shape as the vitamin D_3 pills. The supplemental capsules and placebo were coded by someone other than the investigator before being made available to patients, in order that neither investigator nor patients could be aware of their treatment group. At the end of the study, fasting blood was taken from the subjects. Physical activity levels were determined by interview, using the International Physical Activity Questionnaire (IPAQ). Frequency and duration of physical activity were then expressed as metabolic equivalent of tasks (METs) [23] and divided in 3 groups, low (0–599), moderate(600–2999) and high($\leq$3000).

2.1. Vitamin D assessment

In order to ensure that the diet did not change during the study, at the beginning of the study, and at the end of the first month, the participants were instructed to fill in a 3-nonconsecutive-day food record, with one of the days being on the weekend. Nutritionist 4 software was used to obtain information concerning vitamin D intake. Moreover, all participants completed a validated questionnaire that included questions about the amount and duration of skin sun exposure and vitamin D supplement consumption at the beginning of study and at end of Eighth week.

2.2. Biochemical assays

Fasting blood samples were collected from all patients at their entry to the study and eight weeks after intervention. Serum was centrifuged, aliquoted and stored at a temperature of −80 °C. Patients routinely received clinical blood work to assess their biochemical parameters. The blood pressure of all participants was measured before and after intervention. Systolic and diastolic blood pressure was measured from the right arm after at least 5 min' rest. GOD/PAP method was used for the measurement of FBS, Serum creatinine and calcium were measured using the Spectrophotometric Method and GFR using the Cockcroft-Gault Equation. Hemoglobin A1c (HbA1c) was measured using ion-exchange chromatography. Insulin concentration, serum levels of TNF-α and IL-6 and 25(OH)D_3 were measured using ELISA-specific kits by the ELISA method. HOMA–IR was calculated using the following equation: HOMA–IR=Fasting insulin (μU/mL) x Fasting glucose (mg/dL)/405 [24].

Table 1
Characteristics of study subjects before intervention.

Variables		Study groups		P value*
		Intervention (n=25)	Placebo (n=25)	
Sex	Male	16(64)	11(44)	0.12
	Female	9(36)	14(56)	
Sun exposure	10–60 min/day	10(40)	8(32)	0.49
	60–120 min/day	12(48)	11(52)	
	higher	3(12)	6(24)	
Physical Activity (METs)	Low	14(56)	7(28)	0.018¥
	Moderate	8(32)	13(52)	
	High	3(12)	5(20)	

*P value was reported based on Chi-Square test. ¥ $P < 0.05$.

2.3. Statistical analysis

Kolmogorov–Smirnov test was conducted to evaluate the normal distribution of quantitative variables. Normally distributed continuous variables were expressed as the mean ± SD. Statistical analysis software SPSS v.21 was used to analyze data. Paired sample *t*-test was conducted to compare means before and after intervention of the treatment and placebo groups for each variable. In order to comparie between treatment and placebo groups, ANCOVA analyses were used for adjusting by baseline value and physical activity. The values were expressed as mean ± standard deviation. Statistical significance was defined as $P< 0.05$ for all tests.

2.4. Ethical consideration

The study protocol followed the principals of the "Declaration of Helsinki". The participants were told that they could withdraw from the study at any time and that all information would be kept secret and anonymous. The required permissions for research were obtained from the vice chancellor of Tabriz University of Medical Sciences with ethics number TBZMED.REC.1394.682. Written and informed consents were obtained from all the participating in the study.

3. Results

The number of subjects included in this study was 50. There were 25 subjects each in the intervention and placebo groups. Mean age, weight and height for vitamin D group was (39.7 ± 7.3), (74.91 ± 1.1) and (167.1 ± 8.2), moreover for placebo group was (43.7 ± 6.1), (72.1 ± 11.6) and (167.3 ± 8.0), respectively. The distribution of the study population based on sex, sun exposure and physical activity is given in Table 1. On the basis of sex and sun exposure distribution, no significant differences were observed between the two groups. However, there was a significant difference of physical activity between the intervention and placebo groups ($P = 0.018$). Energy adjusted dietary intake of study patient at baseline and endpoint is demonstrated in Table 2. There is no significant different for intake of macro and micronutrients in baseline and endpoint of study in both placebo and vitamin D groups.

The results of metabolic and inflammatory parameters analysis are shown in Table 3. The analysis showed a significant increase of blood level vitamin D after treatment only among the intervention group ($P < 0.001$) while significant differences were found in vitamin D levels between the intervention and placebo groups ($p < 0.001$). There were no significant differences between the percentage changes of HbA1c, insulin, and inflammatory parameters such as TNF-α and IL-6 ($P > 0.05$) between two groups, while the percentage change of FBS was significantly higher in the placebo group compared to the treatment group ($P < 0.0001$). This remained significant even after adjusting for physical activity. Moreover, when the before and after differences were evaluated for each group, lower levels of FBS ($P < 0.0001$), insulin ($P < 0.069$), HOMA-IR ($P < 0.001$), TNF-α ($P < 0.002$) and IL-6 ($P < 0.037$) were found after supplementation in the treatment group, but no significant differences were found between the placebo group. Moreover, there were no significant differences for weight before and after intervention for both placebo ($P = 0.183$) and intervention ($p = 0.267$) groups. Also systolic ($P < 0.0001$) and diastolic ($P < 0.0001$) blood pressure was significantly lower after intervention for placebo group but not for intervention group, $P = 0.341$ and 0.226 respectively.

A comparison of the mean blood and urine parameters between two groups of the study is shown in Table 4. As shown, there were no significant differences of creatinine, calcium, albumin and GFR

Table 2
Dietary intake of study patients at baseline and endpoint.

	Vitamin D group (n = 25)			Placebo group (n = 25)		
	baseline	endpoint	p	baseline	endpoint	p
Energy (kcal/d)	1560 ± 550	1607 ± 532	0.42	1594 ± 567	1628 ± 546	0.53
Carbohydrate (g/d)	188.7 ± 81	192.3 ± 83	0.91	201.4 ± 97	204.5 ± 78	0.68
Protein (g/d)	57.8 ± 12.7	60.7 ± 14.4	0.46	59.6 ± 12.9	62.6 ± 11.1	0.49
Total fat (g/d)	57.9 ± 15	59.5 ± 16.2	0.57	62.6 ± 14.7	63.0 ± 15.3	0.61
Fiber (g/d)	16.4 ± 7.4	17.6 ± 3.6	0.32	15.5 ± 6.3	16.6 ± 5.4	0.63
Vitamin D (μg/d)	2.2 ± 0.7	2.1 ± 0.4	0.61	2.3 ± 0.3	2.2 ± 0.8	0.57
Vitamin E (mg/d)	21.6 ± 5.8	19.3 ± 5.6	0.53	20.32 ± 6.1	19.73 ± 5.3	0.67
Vitamin C (mg/d)	155.4 ± 98.2	140.3 ± 101.2	0.43	127.6 ± 92.3	112.1 ± 76.2	0.52
Calcium (g/d)	1.13 ± 0.13	1.21 ± 0.21	0.43	1.24 ± 0.25	1.19 ± 0.17	0.48
Zinc (mg/d)	12.5 ± 5.2	13.8 ± 6.6	0.72	13.2 ± 6.2	12.9 ± 6.7	0.64
Iron (mg/d)	18.3 ± 4	19.6 ± 6	0.31	17.7 ± 7.2	16.3 ± 5.4	0.42
Phosphorus (g/d)	1.17 ± 0.18	1.20 ± 0.15	0.59	1.15 ± 0.19	1.21 ± 0.19	0.73

Values are means ± standard deviation. *P* value is reported based on the analysis of paired sample *t*-test.

Table 3
Metabolic and Inflammatory parameters.

		Groups				P value*	P value**
		Intervention (n = 25)		Placebo (n = 25)			
		Mean ± SD	PC	Mean ± SD	PC		
Vitamin D (ng/ml)	**Before**	21.67 ± 5.62	84.58	22.36 ± 5.70	7.84	<0.001	<0.001
	After	37.63 ± 7.73		24.11 ± 7.62			
	P-Value	<0.001		0.08			
Diabetic Parameters							
HbA1c (mmol/l)	**Before**	6.36 ± 1.11	−1.17	6.61 ± 1.46	−4.9	0.66	0.32
	After	6.26 ± 1.58		6.16 ± 1.40			
	P-Value	0.79		0.13			
FBS (mg/dl)	**Before**	129.12 ± 11.8	−12.40	129.84 ± 10.42	1.34	<0.0001¥	<0.0001¥
	After	111.86 ± 13.61		130.99 ± 11.79			
	P-Value	<0.0001¥		0.69			
HOMA-IR	**Before**	11.50 ± 3.86	−16.38	11.45 ± 3.41	2.62	0.001¥	0.002¥
	After	9.31 ± 2.97		11.64 ± 4.20			
	P-Value	<0.001¥		0.736			
Insulin (mIU/L)	**Before**	36.11 ± 11.48	−3.39	35.78 ± 10.33	0.71	0.27	0.15
	After	34.23 ± 11.26		35.68 ± 10.80			
	P-Value	0.069		0.94			
Inflammatory Parameters							
TNF-α (pg/ml)	**Before**	136.6 ± 33.65	−10.97	133.22 ± 29.32	0.32	0.34	0.094
	After	118.47 ± 27.58		129.57 ± 22.51			
	P-Value	0.002¥		0.43			
IL-6 (pg/ml)	**Before**	114.38 ± 33.17	−5.38	113.17 ± 27.17	0.19	0.122	0.124
	After	103.28 ± 25.07		110.76 ± 20.13			
	P-Value	0.037¥		0.53			

*P-value reported based on Paired Sample T test. ** P-value reported based on ANCOVA after baseline value adjustment. © P-value reported based on ANCOVA after adjustment of baseline values and physical activity. PC: percent change, ¥ P < 0.05.

between the intervention and placebo groups (P > 0.05). On the other hand, the phosphorous and protein percentage changes in urine were lower (P = 0.07) and higher (P = 0.003) among both groups, respectively. After adjustment for physical activity, only the protein level remained significant. The analysis showed lower levels of urinary protein (P= 0.001) only in the intervention group.

4. Discussion

The aim of this study was to assess the effect of vitamin D supplementation on metabolic and inflammatory parameters in diabetic nephropathy patients. In this study, the vitamin D intake group showed a significant decrease in urinary protein and FBS levels compared to the placebo group, but this had no significant effect on inflammatory parameters such as TNF-α and IL-6 after intervention. There was some evidence which showed that the variation in vitamin D blood levels had a relationship with the risk factors of chronic diseases. The effects of vitamin D supplementation on the levels of FBS have been demonstrated in previous studies.

According to our analyses, FBS levels were significantly lower after the intervention among nephropathy diabetic patients. The association between HOMA-IR, FBS blood levels and vitamin D has been previously demonstrated in different diseases, such as

Table 4
Blood and urine parameters.

		Groups				P value*	P value**
		Intervention (n = 25)		Placebo (n = 25)			
		Mean ± SD	PC	Mean ± SD	PC		
Cr(μg/ml)	**Before**	1.31 ± 0.33	15.41	1.15 ± 0.36	46.34	0.25	0.59
	After	1.13 ± 0.66		1.31 ± 0.53			
	P-Value	0.95		0.35			
Ca(mg/dl)	**Before**	8.29 ± 1.05	5.22	7.99 ± 1.08	7.67	0.14	0.62
	After	8.56 ± 1.16		8.50 ± 1.14			
	P-Value	0.40		0.1			
P(mg/dl)	**Before**	3.06 ± 0.7	22.47	2.88 ± 0.74	12.07	0.07	0.11
	After	3.56 ± 1.27		3.08 ± 0.47			
	P-Value	0.09		0.26			
Alb (g/l)	**Before**	46.70 ± 17.10	8.33	49.35 ± 15.61	1.44	0.82	0.70
	After	47.86 ± 17.59		48.48 ± 12.93			
	P-Value	0.72		0.71			
Pr (urine) (mg/dl)	**Before**	333.80 ± 112.96	−23.70	325.38 ± 110.75	1.47	0.03	0.006
	After	233.96 ± 118.75		319.91101.91			
	P-Value	0.001		0.72			
GFR (ml/min/1.73^2)	**Before**	45.87 ± 17.39	0.92	46.97 ± 12.26	1.13	0.96	0.81
	After	46.96 ± 12.17		46.46 ± 13.64			
	P-Value	0.58		0.89			

*P-value reported based on Paired Sample T test. ** P-value reported based on ANCOVA after baseline value adjustment. © P-value reported based on ANCOVA after adjustment of baseline values and physical activity. PC: percent change.

metabolic syndrome [25,26] and diabetes type 2 [27]. A previous study done by Kaviani M et al. found that vitamin D supplementation does not effect HOMA-IR, whereas Talaei A et al. [27] reported significant improvements in serum HOMA-IR after vitamin D supplementation. Gulseth et al. [28]showed the significant role of vitamin D in the stimulation of insulin secretion or sensitivity. The effect of this vitamin on insulin sensitivity appears to be due to the effect on stimulating the expression of the insulin receptor gene [16]. Diaz et al. [29] found a significant relationship between lower serum level vitamin D and a higher risk of nephropathy among diabetic patients. In another study, Li et al. [18] showed that the prevalence of nephropathy in patients with vitamin D deficiency was higher than those with normal levels of vitamin D, while the prevalence of proteinuria in patients with vitamin D deficiency increased. In a recent study by Usluogullari et al. [30], a positive relationship between the severity of vitamin D deficiency and nephropathy was shown. In contrast to the current study, Kajbaf et al. [31] indicated a significant inverse relationship between vitamin D and HbA1c.

It was also found that vitamin D supplementation had no significant effect on IL-6 and TNF-α levels in the intervention group, compared to the placebo group, but in the intervention group the level of this cytokine decreased after vitamin D supplementation. It has been suggested that vitamin D deficiency is associated with increased levels of inflammatory markers, particularly IL-6. Manion et al. [32] suggested that patients with vitamin D deficiency had 23% higher IL-6 levels compared to those with normal vitamin D levels. However, in contrast to the present study, Naghavi et al. [33] demonstrated that vitamin D supplementation may up-regulate the gene expression of IL-6. Moreover, Nameini et al. [34] demonstrated that Vitamin D had no effect on TNF-α. Documented evidence, though, has shown vitamin D to reduce inflammatory cytokines directly. It decrease the level of TNF-α and IL-6 through inhibiting their production in EOC13 microglial cells [35]. Due to the role of TNF-α in the stimulation of renal micro-inflammation, it may affect the process of pro-inflammatory molecule production during the progression of diabetic nephropathy [36]. Therefore, it seems that vitamin D supplementation may reduce diabetic nephropathy progression through down-regulating pro-inflammatory molecule production, such as TNF-α and IL-6.

The present study showed there was no significant change in serum creatinine, calcium, phosphorus and albumin before and after intervention among both groups. In contrast with these results, Agarwal R et al. [37], who worked on the effect of VDR (vitamin D receptor) activation on serum creatinine, found increased VDR activation correlated with increased creatinine levels, but had no influence on GFR. Similar results were found by Bentli R et al. [38], who reported that the intake of vitamin D supplementation had increased effects on creatinine levels among nephropathy patients. Agarwal et al. suggest short-term vitamin D receptor activation may increase creatinine level but this increase in serum creatinine may be related to the augmented release of creatinine from muscular tissue [37]. A recent study done by Xiao X et al. [39] showed that as diabetic nephropathy progresses, levels of calcium and albumin are reduced in line with decreases in levels of vitamin D.

Although GFR had no significant change in both the placebo and intervention groups before and after supplementation, proteinuria significantly decreased in patients who received vitamin D supplementation. Findings from several studies have indicated that vitamin D has reduced proteinuria in patients with a different range of diseases, such as diabetes and CKD (Chronic Kidney Disease) [40,41]. Several mechanisms have been suggested in previous mechanistic studies. Vitamin D may down-regulate the expression of the pro-renin gene, and also may affect other renin-angiotensin-aldosterone system (RAAS) components [42] [43]. The inhibition of the TGF-β pathway was also another suggested mechanism to reduce renal dysfunction and proteinuria [42]. It seems that reduced proteinuria can be considered as an important strategy to prevent or postpone diabetic nephropathy progression in type 2 diabetes patients [44]. Hence, vitamin D supplementation may have a beneficial influence on diabetic nephropathy progression through its effects on reduced proteinuria.

There are some limitations in the current study that need to be taken into account. Despitecontrolling for a wide range of potential confounders, the effects of residual confounders cannot be excluded. Moreover, we didn't vitamin D binding protein -which is suggested as a novel biomarker to predict diabetic nephropathy.

5. Conclusion

In conclusion, evidence was found indicating that vitamin D supplementation can be regarded as an effective way to prevent the progression of diabetic nephropathy, due to its beneficial influence on reduced levels of proteinuria and some inflammatory markers such as TNF-α and IL-6.

Authors' contribution

Study concept and design: Pourghassem Gargari, Esfandiari; acquisition of data: Noshad, Mobasseri, Esfandiari, Barzegari; analysis and interpretation of data: Pourghassem Gargari; drafting of the manuscript: Esfandiari; critical revision of the manuscript for intellectual content: Pourghassem Gargari: statistical analysis, administrative, technical, and material support: Sarbakhsh, Arzhang; study supervision: Pourghassem Gargari.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsx.2018.09.013.

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